TRANSLATOR'S DECLARATION

I, Janet Hope, BSc (Hons.), MIL., MITI., translator to Messrs. Taylor and Meyer of 20 Kingsmead Road, London, SW2 3JD, Great Britain, verify that I know well both the German and the English language, that I have prepared the attached English translation of 28 pages of a German Patent application in the German language with the title:

Neue für das metF-Gen kodierende Nukleotidsequenzen

identified by the code number 000363 BT at the upper left of each page and corresponding to client/matter number of the law firm of

and that the attached English translation of this document is a true and correct translation of the document attached thereto to the best of my knowledge and belief.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of this document.

By: _____ Date: 16 October 2003

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The attached papers are a true and accurate reproduction of the original documents for this patent application.

Munich, 7th June 2001
On behalf of the President of the German Patent and Trade Mark Office

(signature)

Wehner

New nucleotide sequences which code for the metF gene

The invention provides nucleotide sequences from coryneform bacteria which code for the metF gene and a process for the fermentative preparation of amino acids, in particular L-methionine, using bacteria in which the metF gene is enhanced.

Prior art

L-Amino acids, in particular L-methionine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the methionine analogue α-methyl-methionine, ethionine, norleucine, N-acetylnorleucine, S-trifluoromethylhomocysteine, 2-amino-5-heprenoitic acid, seleno-methionine, methionine-sulfoximine, methoxine, 1-aminocyclopentane-carboxylic acid, or are auxotrophic for metabolites of regulatory

importance and produce amino acid, such as e.g. L-methionine, are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium strains which produce L-amino acid, by amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

Object of the invention

The inventors had the object of providing new measures for improved fermentative preparation of amino acids, in particular L-methionine.

Description of the invention

When L-methionine or methionine are mentioned in the following, the salts, such as e.g. methionine hydrochloride or methionine sulfate are also meant by this.

The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the metF gene, chosen from the group consisting of

a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,

b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of methylene tetrahydrofolate reductase.

The invention also provides the abovementioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

(i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or

(iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally

(iv) sense mutations of neutral function in (i).

The invention also provides

a polynucleotide comprising the nucleotide sequence as shown in SEQ ID No. 1,

a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2,

a vector containing the polynucleotide according to the invention, in particular a shuttle vector or plasmid vector, and

coryneform bacteria serving as the host cell, which contain the vector or in which the metF gene is enhanced.

The invention also provides polynucleotides which substantially comprise a polynucleotide sequence, which are

obtainable by screening by means of hybridization of a corresponding gene library, which comprises the complete gene with the polynucleotide sequence corresponding to SEQ ID No. 1, with a probe which comprises the sequence of the polynucleotide mentioned, according to SEQ ID No. 1 or a fragment thereof, and isolation of the DNA sequence mentioned.

Polynucleotide sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for methylene tetrahydrofolate reductase or to isolate those nucleic acids or polynucleotides or genes which have a high similarity of sequence to methylene tetrahydrofolate reductase.

Polynucleotide sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for methylene tetrahydrofolate reductase can be prepared by the polymerase chain reaction (PCR).

Such oligonucleotides which serve as probes or primers comprise at least 30, preferably at least 20, very particularly preferably at least 15 successive nucleotides. Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

"Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of methylene tetrahydrofolate reductase, and also those which are at least 70%, preferably at least 80% and in particular which are at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

The invention moreover provides a process for the fermentative preparation of amino acids, in particular L-methionine, using coryneform bacteria which in particular already produce amino acids, and in which the nucleotide sequences which code for the metF gene are enhanced, in particular over-expressed.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene which codes for a corresponding enzyme having a high activity, and optionally combining these measures.

The microorganisms which the present invention provides can prepare L-amino acids, in particular L-methionine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus Corynebacterium. Of the genus Corynebacterium, there may be mentioned in particular the species Corynebacterium glutamicum, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum (C. glutamicum), are in particular the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

or L-amino acid-producing mutants or strains prepared therefrom, such as, for example, the L-methionine-producing strain

Corynebacterium glutamicum ATCC21608.

The inventors succeeded in isolating the new metF gene from C. glutamicum which codes for the enzyme methylene tetrahydrofolate reductase [EC:1.7.99.5].

To isolate the metF gene or also other genes of C. glutamicum, a gene library of this microorganism is first set up in Escherichia coli (E. coli). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990), or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989) may be mentioned as an example. A well-known gene library is that of the E. coli K-12 strain W3110 set up in λ vectors by Kohara et al. (Cell 50, 495 - 508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of C. glutamicum ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992)) in turn describe a gene library of C. glutamicum ATCC13032 using the cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980)).

To prepare a gene library of C. glutamicum in E. coli it is also possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are, in particular, those E. coli strains which are restriction- and recombination-defective. An example of these is the strain DH5αmcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can in turn be subcloned in the usual vectors suitable for sequencing and then sequenced, as is described e.g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

In this way, the new DNA sequence of C. glutamicum which codes for the metF gene and which, as SEQ ID No. 1, is a constituent of the present invention was obtained. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the metF gene product is shown in SEQ ID No. 2.

Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which

hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function.

It is furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonucleotide synthesis: A Practical Approach (IRL Press,

Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It has been found that coryneform bacteria produce amino acids, in particular L-methionine, in an improved manner after over-expression of the metF gene.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-methionine production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European Patent Specification 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15 - 24 (1993)), in Japanese Laid-Open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and

Bioengineering 58, 191-195 (1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks of genetics and molecular biology.

By way of example, for enhancement the metF gene according to the invention was over-expressed with the aid of episomal plasmids. Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors, such as e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as e.g. those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A 5,158,891), can be used in the same manner.

Plasmid vectors which are furthermore suitable are also those with the aid of which the process of gene amplification by integration into the chromosome can be used, as has been described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for duplication or amplification of the hom-thrB operon. In this method, the complete gene is cloned in a plasmid vector which can replicate in a host (typically E. coli), but not in C. glutamicum. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al.,1986, Gene 41: 337-342). The plasmid vector which contains the gene to be amplified is then transferred into

the desired strain of C. glutamicum by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene in question.

In addition, it may be advantageous for the production of amino acids, in particular L-methionine, to enhance one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle or of amino acid export, in addition to the metF gene.

Thus for the preparation of amino acids for example, in particular L-methionine, one or more genes chosen from the group consisting of

- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pyc gene which codes for pyruvate carboxylase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the lysC gene which codes for a feed-back resistant aspartate kinase (ACCESSION NUMBER P26512),

- the metA gene which codes for homoserine O-acetyltransferase (ACCESSION Number AF052652),
- the metB gene which codes for cystathionine gamma-synthase (ACCESSION Number AF126953),
- the aecD gene which codes for cystathionine gamma-lyase (ACCESSION Number M89931)
- the glyA gene which codes for serine hydroxymethyltransferase (JP-A-08107788),
- the metY gene which codes for O-acetylhomoserine sulfhydrylase (DSM 13556)

can be enhanced, in particular over-expressed.

It may furthermore be advantageous for the production of amino acids, in particular L-methionine, in addition to the enhancement of the metF gene, for one or more genes chosen from the group consisting of

- the thrB gene which codes for homoserine kinase (ACCESSION Number P08210),
- the ilvA gene which codes for threonine dehydratase (ACCESSION Number Q04513),
- the thrC gene which codes for threonine synthase (ACCESSION Number P23669),
- the ddh gene which codes for meso-diaminopimelate D-dehydrogenase (ACCESSION Number Y00151),
- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478; DSM 12969),

- the poxB gene which codes for pyruvate oxidase (DE: 1995 1975.7; DSM 13114)

to be attenuated, in particular for the expression thereof to be reduced.

In addition to over-expression of the metF gene it may furthermore be advantageous, for the production of amino acids, in particular L-methionine, to eliminate undesirable side reactions, (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms prepared according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of amino acids, in particular L-methionine. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols,

such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued

until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-methionine can be carried out by ion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190).

The process according to the invention is used for the fermentative preparation of amino acids, in particular L-methionine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

Example 1

Preparation of a genomic cosmid gene library from Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al.

(1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 2

Isolation and sequencing of the metF gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A.,

87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

The plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig. The computer-assisted coding region analysis was prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence showed an open reading frame of 1046 base pairs, which was called the metF gene. The metF gene codes for a protein of 349 amino acids.

SEQUENCE PROTOCOL

<110> Degussa-Hüls AG

<120> New nucleotide sequences which code for the metF gene

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<170> PatentIn Ver. 2.1

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<222> (299)..(1345)
<223> metF gene

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tgggccacag catctgtgac aggagcgccg atatcagcaa ttgttagctc ttgagcatcg 180

aggaactgcg tcaaacgatc tcgcacgacc tccggaaatt tgtcgaggtc aaggtcatgg 240

gcatcgaaac tgctcaagga gacgtccttc aatcgaatag ggggatgcgg gctgaatt 298

ttg gtg gag gtg aat aaa tgc cag agg cag tcc caa caa aac act ctc 346
Leu Val Glu Val Asn Lys Cys Gln Arg Gln Ser Gln Gln Asn Thr Leu
1 5 10 15

atc aca cta aga tac cca ggc atg tcc cta acg aac atc cca gcc tca 394
Ile Thr Leu Arg Tyr Pro Gly Met Ser Leu Thr Asn Ile Pro Ala Ser
20 25 30

tct caa tgg gca att agc gac gtt ttg aag cgt cct tca ccc ggc cga 442
Ser Gln Trp Ala Ile Ser Asp Val Leu Lys Arg Pro Ser Pro Gly Arg
35 40 45

gta cct ttt tct gtc gag ttt atg cca ccc cgc gac gat gca gct gaa 490
Val Pro Phe Ser Val Glu Phe Met Pro Pro Arg Asp Asp Ala Ala Glu
50 55 60

gag cgt ctt tac cgc gca gca gag gtc ttc cat gac ctc ggt gca tcg 538
Glu Arg Leu Tyr Arg Ala Ala Glu Val Phe His Asp Leu Gly Ala Ser
65 70 75 80

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Ser Arg Ile Ala Arg Arg Leu Ala Lys Gln Pro Leu Thr Thr Leu Val
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His Leu Thr Leu Val Asn His Thr Arg Glu Glu Met Lys Ala Ile Leu
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cgg gaa tac cta gag ctg gga tta aca aac ctg ttg gcg ctt cga gga   730
Arg Glu Tyr Leu Glu Leu Gly Leu Thr Asn Leu Leu Ala Leu Arg Gly
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gat ccg cct gga gac cca tta ggc gat tgg gtg agc acc gat gga gga   778
Asp Pro Pro Gly Asp Pro Leu Gly Asp Trp Val Ser Thr Asp Gly Gly
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ctg aac tat gcc tct gag ctc atc gat ctt att aag tcc act cct gag   826
Leu Asn Tyr Ala Ser Glu Leu Ile Asp Leu Ile Lys Ser Thr Pro Glu
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ttc cgg gaa ttc gac ctc ggt atc gcc tcc ttc ccc gaa ggg cat ttc   874
Phe Arg Glu Phe Asp Leu Gly Ile Ala Ser Phe Pro Glu Gly His Phe
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cgg gcg aaa act cta gaa gaa gac acc aaa tac act ctg gcg aag ctg   922
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cgt gga ggg gca gag tac tcc atc acg cag atg ttc ttt gat gtg gaa   970
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Gly Ala Lys Pro Ile Ile Pro Gly Ile Met Pro Ile Thr Glu Leu Arg
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tct gtg cgt cga cag gtc gaa ctc tct ggt gct caa ttg ccg agc caa   1114
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Leu Glu Glu Ser Leu Val Arg Ala Ala Asn Gly Asn Glu Glu Ala Asn
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aaa gac gag atc cgc aag gtg ggc att gaa tat tcc acc aat atg gca   1210
Lys Asp Glu Ile Arg Lys Val Gly Ile Glu Tyr Ser Thr Asn Met Ala
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gag cga ctc att gcc gaa ggt gcg gaa gat ctg cac ttc atg acg ctt   1258
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Pro Ala Trp Gly Ala Glu His Gly Gln Asp Ala Val Arg
340 345

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Ser Gln Trp Ala Ile Ser Asp Val Leu Lys Arg Pro Ser Pro Gly Arg
35 40 45

Val Pro Phe Ser Val Glu Phe Met Pro Pro Arg Asp Asp Ala Ala Glu
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Glu Arg Leu Tyr Arg Ala Ala Glu Val Phe His Asp Leu Gly Ala Ser
65 70 75 80

Phe Val Ser Val Thr Tyr Gly Ala Gly Gly Ser Thr Arg Glu Arg Thr
85 90 95

Ser Arg Ile Ala Arg Arg Leu Ala Lys Gln Pro Leu Thr Thr Leu Val
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His Leu Thr Leu Val Asn His Thr Arg Glu Glu Met Lys Ala Ile Leu
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Arg Glu Tyr Leu Glu Leu Gly Leu Thr Asn Leu Leu Ala Leu Arg Gly
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Asp Pro Pro Gly Asp Pro Leu Gly Asp Trp Val Ser Thr Asp Gly Gly
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Leu Asn Tyr Ala Ser Glu Leu Ile Asp Leu Ile Lys Ser Thr Pro Glu
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Phe Arg Glu Phe Asp Leu Gly Ile Ala Ser Phe Pro Glu Gly His Phe
180 185 190

Arg Ala Lys Thr Leu Glu Glu Asp Thr Lys Tyr Thr Leu Ala Lys Leu
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Arg Gly Gly Ala Glu Tyr Ser Ile Thr Gln Met Phe Phe Asp Val Glu
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Asp Tyr Leu Arg Leu Arg Asp Arg Leu Val Ala Ala Asp Pro Ile His
225 230 235 240

Gly Ala Lys Pro Ile Ile Pro Gly Ile Met Pro Ile Thr Glu Leu Arg
245 250 255

Ser Val Arg Arg Gln Val Glu Leu Ser Gly Ala Gln Leu Pro Ser Gln
260 265 270

Leu Glu Glu Ser Leu Val Arg Ala Ala Asn Gly Asn Glu Glu Ala Asn
275 280 285

Lys Asp Glu Ile Arg Lys Val Gly Ile Glu Tyr Ser Thr Asn Met Ala
290 295 300

Glu Arg Leu Ile Ala Glu Gly Ala Glu Asp Leu His Phe Met Thr Leu
305 310 315 320

Asn Phe Thr Arg Ala Thr Gln Glu Val Leu Tyr Asn Leu Gly Met Ala
325 330 335

Pro Ala Trp Gly Ala Glu His Gly Gln Asp Ala Val Arg
340 345

Patent claims

1. An isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence chosen from the group consisting of

   a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,

   b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

   c) polynucleotide which is complementary to the polynucleotides of a) or b), and

   d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c).

2. A polynucleotide as claimed in claim 1, wherein the polynucleotide is a preferably recombinant DNA which is capable of replication in coryneform bacteria.

3. A polynucleotide as claimed in claim 1, wherein the polynucleotide is an RNA.

4. A polynucleotide as claimed in claim 2, comprising the nucleic acid sequence as shown in SEQ ID No. 1.

5. A DNA as claimed in claim 2 which is capable of replication, comprising

   (i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or

(iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally

(iv) sense mutations of neutral function in (i).

6. A polynucleotide sequence as claimed in claim 2, which codes for a polypeptide which comprises the amino acid sequence in SEQ ID No. 2.

7. A coryneform bacterium in which the metF gene is enhanced, in particular over-expressed.

8. A coryneform bacterium serving as the host cell, which contains a vector which carries a polynucleotide as claimed in claim 1.

9. A process for the fermentative preparation of L-amino acids, in particular L-methionine, which comprises carrying out the following steps:

a) fermentation of the coryneform bacteria which produce the desired L-amino acid and in which at least the metF gene or nucleotide sequences which code for it are enhanced, in particular over-expressed;

b) concentration of the L-amino acid in the medium or in the cells of the bacteria, and

c) isolation of the L-amino acid.

10. A process as claimed in claim 9, wherein bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.

11. A process as claimed in claim 9, wherein bacteria in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.

12. A process as claimed in claim 9, wherein a strain transformed with a plasmid vector is employed, and the plasmid vector carries the nucleotide sequence which codes for the metF gene.

13. A process as claimed in claim 9, wherein the expression of the polynucleotide(s) which code(s) for the metF gene is enhanced, in particular over-expressed.

14. A process as claimed in claim 9, wherein the catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide metF codes are increased.

15. A process as claimed in claim 9, wherein for the preparation of L-amino acids, in particular L-methionine, coryneform microorganisms in which at the same time one or more of the genes chosen from the group consisting of

15.1 the lysC gene which codes for a feed back resistant aspartate kinase,

15.2 the gap gene which codes for glycerolaldehyde 3-phosphate dehydrogenase,

15.3 the pgk gene which codes for 3-phosphoglycerate kinase,

15.4 the pyc gene which codes for pyruvate carboxylase,

15.5 the tpi gene which codes for triose phosphate isomerase

15.6 the metA gene which codes for homoserine O-acetyltransferase

15.7 the metB gene which codes for cystathionine gamma-synthase

15.8 the aecD gene which codes for cystathionine gamma-lyase

15.9 the glyA gene which codes for serine hydroxymethyltransferase

15.10 the metY gene which codes for O-acetylhomoserine sulfhydrylase

is or are amplified or over-expressed are fermented.

16. A process as claimed in claim 9, wherein for the preparation of L-amino acids, in particular L-methionine, coryneform microorganisms in which at the same time one or more of the genes chosen from the group consisting of

16.1 the thrB gene which codes for homoserine kinase

16.2 the ilvA gene which codes for threonine dehydratase

16.3 the thrC gene which codes for threonine synthase

16.4 the ddh gene which codes for meso-diaminopimelate D-dehydrogenase

16.5 the pck gene which codes for phosphoenol pyruvate carboxykinase

16.6 the pgi gene which codes for glucose 6-phosphate isomerase

16.7 the poxB gene which codes for pyruvate oxidase

is or are attenuated are fermented.

17. A process as claimed in one or more of the preceding claims, wherein microorganisms of the species Corynebacterium glutamicum are employed.

18. A process for discovering RNA, cDNA and DNA in order to isolate nucleic acids, or polynucleotides or genes which code for methylene tetrahydrofolate reductase or have a high similarity with the sequence of the methylene tetrahydrofolate reductase gene, which comprises employing the polynucleotide sequences as claimed in claim 1, 2, 3 or 4 as hybridization probes.

New nucleotide sequences which code for the metF gene

Abstract

The invention relates to an isolated polynucleotide comprising a polynucleotide sequence chosen from the group consisting of

a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,

b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

and processes for the fermentative preparation of L-amino acids using coryneform bacteria in which at least the metF gene is present in enhanced form, and the use of the polynucleotide sequences as hybridization probes.